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## STATUS REPORT

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Robert J. DeLorenzo, M.D., Ph.D., M.P.H. - Principal Investigator

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### ABSTRACT

Hydrazines (HZ) are toxic compounds which have numerous industrial applications including their use as missile propellants in advanced aircraft such as the F-16 and space vehicles. The extremely high toxicity of HZs and the recurrent accidental exposure due to routine storage, use, and disposal of these compounds have created a significant health hazard among aerospace and defense industry personnel. HZ exposure can result in lethal complications involving repeated seizures and eventual respiratory collapse. Acute HZ exposure produces repeated tonic-clonic seizures in animals and man due to the strong convulsant properties of these compounds. In order to develop effective therapies for HZ toxicity, it is important to determine the mechanisms by which HZs produce their neuronal excitatory effects. Studies in our laboratory of the electrophysiological effects of HZs have shown that HZ exposure induces spontaneous and evoked epileptiform activity in mammalian hippocampus, recorded *in vitro*. In cultured hippocampal neurons, we have found that HZs decrease the post-burst afterhyperpolarization, a primary postsynaptic mechanism utilized by many types of neurons to terminate bursts, and maintain a check on hyperexcitability. As expected, this AHP reduction by HZs increases the rate of sustained repetitive firing in these neurons, and may be one mechanism contributing to HZ convulsant actions. The overall objective of this study was to describe and pharmacologically characterize HZ-induced epileptiform actions in hippocampus, and to examine the effects of HZs on ion conductances in mammalian hippocampal neurons, in order to provide insight into the mechanisms of HZ toxicity which may underlie the excitatory and epileptogenic properties of these compounds.

We have combined electrophysiological, biophysical, and pharmacological techniques to examine the effects of HZs at both a single neuron and a systems level in mammalian

hippocampus. We have shown that HZs increase neuronal excitability and the rate of neuronal firing, but the molecular mechanism by which HZs produce these effects is not known. We investigated whether HZs produce, directly or indirectly, their excitatory effects by blockade of inhibitory pathways or enhancement of excitatory mechanisms. The overall goal of this project was to coordinate extracellular, intracellular, voltage-clamp, and single-channel studies to determine whether HZs affect specific membrane currents, and to assess the relative contributions of these mechanisms to the epileptogenic actions of HZs. The Short Term Goals of the project focused on a) characterization of the epileptogenic effects of HZs in hippocampal slices and cultures, and b) identification of specific ion conductances affected by HZ exposure in hippocampal pyramidal neurons. The Longer Term Goals focused on coordinating the previous studies to determine whether anticonvulsants and NMDA antagonists can modulate the excitatory and epileptogenic effects of HZ and to begin to elucidate in greater detail the molecular mechanisms by which HZ affects individual voltage- and ligand-gated membrane conductances. The studies included a) characterization of the effects of anticonvulsants on the epileptogenic action of HZ, b) correlation between the effects of HZ on ion conductances in hippocampal neurons with the effects of anticonvulsants and/or NMDA antagonists on similar processes, c) examination of HZ effects on voltage- and ligand-gated conductances at a single channel level, to rigorously characterize the nature of HZ actions on these conductances.

We have been successful in utilizing the hippocampal slice preparation to demonstrate that HZs cause seizures in this preparation.

## RESEARCH OBJECTIVES

### I. CONVULSANT ACTIONS OF HYDRAZINES

#### a) Extracellular Hippocampal Slice Experiments

The mammalian hippocampal slice preparation has been shown by many investigators to have sufficiently intact synaptic circuitry to support epileptiform electrical activity, and has been widely utilized to study cellular mechanisms of epileptogenesis (1-13, reviewed in 14, 15). Although not normally occurring in untreated hippocampal slices, epileptiform electrical activity can be elicited in both CA1 and CA3 pyramidal cell layers by a number of mechanisms, including repetitive electrical stimulation (19-21), variations in concentrations of ions in the extracellular medium (9,11,32,33), and application of various drugs (9,12,13). In recent work involving extracellular stimulation and recording studies in rat hippocampal slices *in vitro*, we found that application of the convulsant hydrazine at a concentration of 0.1% (v/v, hydrazine monohydrate) induced both spontaneous (Figure 1B4) and stimulation-evoked epileptiform discharges (Figure 1B3), followed by cessation of excitability (Figure 1B4). These effects were reversible (Figure 1C4) and concentration dependent, with threshold responses occurring at concentrations between 0.01 and 0.03% (Figure 2). The reversibility and concentration dependence of hydrazine effects suggest that its convulsant action is due to an effect on one or more specific cellular processes involved in the control of neuronal excitability, and not to non-specific results of membrane damage or cell death, both of which are probably irreversible in the time frame of *in vitro* recording. The concentrations found to produce epileptiform activity are comparable to the blood levels of HZ that produce lethal convulsions in animals (24) and man.

Studies centered on rigorous examination of the concentration-dependence of HZ epileptogenic effects, and detailed description and analysis of the electrographic epileptiform

activity induced by HZ exposure to hippocampal slices. As medium-to-long term goals in the hippocampal slice preparation, we plan 1) to record from individual neurons within slices during HZ exposure (in addition to the culture experiments described below) in order to further describe the epileptiform activity elicited by HZs, and 2) to begin to explore the pharmacology of drugs which may block HZ convulsant activity, examining the effects of various anticonvulsants and excitatory amino acid antagonists on HZ-induced epileptiform activity.

#### **b) Intracellular Primary Culture**

Hippocampal neurons in culture, unlike slices, are no longer connected in an ordered synaptic network. However, they are easy to visualize and amenable to high resolution patch-clamp recording. We were therefore interested in determining whether HZs could induce epileptiform activity in cultures, allowing us to extend our model system of HZ convulsant activity to include a preparation permitting rigorous biophysical analysis, simplifying the study of cellular mechanisms involved in generating this activity.

One-to-two week old cultured hippocampal neurons were used in these experiments. These neurons were usually spontaneously active, firing action potentials on a underlying background synaptic barrage. These action potentials could be categorized as large, medium and small amplitude, corresponding to somatic, axonal and dendritic spikes respectively (Figure 3). In preliminary experiments, dropwise application of concentrated HZ stock (final bath concentration of roughly 0.1%), following a delay of 7-10 minutes, induced large increases in spontaneous synaptic activity. This increase in synaptic activity culminated in waves of excitation eliciting bursts of action potentials, often followed by prolonged, 10-20 mV depolarizing plateau potentials. Some bursts were followed by extremely prolonged large (30-40 mV) depolarizations which lasted up to 5 seconds. These large and long lasting waves of depolarization were sometimes followed by 7-10 shorter, closely-spaced bursts, as shown in

Figure 3. In some preparations, shorter bursts appeared as "epileptiform" activity with an ongoing frequency of repetition of about 1 Hz. Each burst consisted of 4-5 subbursts. Each subburst contained 2-5 spikes. These bursts were occasionally interrupted by a short pause of about 1-3 seconds (Figure 3). This type of activity resembles a single cell manifestation of the spontaneous activity elicited by HZs in the hippocampal slice preparation (eg. Figure 1B2). Both the prolonged plateau potentials and extremely prolonged large depolarizations were very rarely recorded under control conditions and never at frequencies approaching those induced by HZs (Figures 3 + 4). Thus, application of HZs to hippocampal cultures produced epileptiform activity, with similar characteristics to the activity induced by HZs in hippocampal slices. In addition, the delay seen between application of HZs and induction of epileptiform activity in slices was also evident in primary culture (Figure 4), suggesting similar processes occur following HZ application in both preparations. Our preliminary results on HZ effects in culture are therefore extremely promising, exhibiting marked resemblance to HZ effects in slice, and substantiating the potential utility of primary hippocampal cultures as a system in which to study cellular mechanisms of action of HZs.

This study examined the concentration dependence of HZ effects and characterized its effects in culture compared to slice, under conditions where HZ bath concentrations are better controlled, without compromising experimenter safety. In our preliminary experiments, we applied HZ stock dropwise to the 2 ml bath volume of the culture dish, to achieve a rough final bath concentration of HZ approximating levels inducing epileptiform activity in slice. This was done to minimize exposure of the researcher to HZs.

## RESEARCH STATUS REPORT

### I. DESCRIPTION OF CELLULAR MECHANISMS OF HZ-INDUCED EPILEPTOGENESIS

#### a) The Effects of HZs on Calcium-Dependent Afterhyperpolarizations and Spike Frequency Adaptation.

In central neurons, one primary mechanism for controlling excitability and terminating bursts is through activation of calcium-dependent afterhyperpolarization potentials (24-31). In addition, AHPs have been implicated in the process of spike frequency adaptation (SFA), an important mechanism controlling neuronal excitability and regulating repetitive firing (32). There are two major types of AHPs: 1) postspike and 2) postburst AHPs. Various types of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  current ( $I_{\text{KCa}^{++}}$ ) have been shown to generate both spike and burst AHPs (33-35). The postspike AHP has been demonstrated in several cell types to be primarily generated by activation of small  $\text{K}^{+}$  (SK) and/or big  $\text{K}^{+}$  (BK) channels (36, 37; reviewed in 38 and 39). In addition to these conductances, postburst AHPs can also be generated by an additional  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  current ( $I_{\text{AHP}}$ ), distinct from SK and BK (34, 35).

We were interested in whether the HZ-induced increase in epileptiform activity and neuronal excitability in both hippocampal slices and cultures might be secondary to an effect of HZs on AHPs. We chose spinal cord neuron cultures for preliminary experiments examining HZ modulation of AHP and SFA. We, along with others, have previously demonstrated that spinal neurons possess calcium-dependent AHPs (40, 41). Unlike cultured hippocampal neurons, spinal neurons are not spontaneously active, and therefore are useful for studying HZs effects on AHP, SFA, and spike shape, since the baseline activity is lower, reducing background noise and increasing the signal-to-noise ratio in our recordings. Even in older cultures, spinal neurons do not make synaptic contacts necessary to form a neuronal network which can produce epileptiform activity during HZ application.



In preliminary experiments, 3-6 week old cultured spinal neurons were used to study HZ effects on postspike and postburst AHPs, as well as on SFA. Various concentrations of HZs were applied to the neurons in the culture dish dropwise to minimize exposure to the experimenter via evaporation (final bath concentration roughly 0.1%). The first observable effect, as in our slice and hippocampal culture experiments, usually occurred about 7-10 minutes following HZ application. HZ application decreased the amplitude and the duration of postspike AHP, resulting in a broadening of the action potential from 3-4 msec duration to about 10 msec (Figure 5A). HZ also affected responses of spinal neurons to sustained stimuli. Under control conditions, the frequency of spike firing in spinal neurons decreased markedly with time during a sustained depolarization. This spike frequency adaptation (SFA) was reduced by HZ application. Figure 5B + C shows an increase in the number of spikes fired 10 minutes after HZ application (a *decrease* in SFA), compared to the number of spikes elicited by an identical depolarization under control conditions in the same neuron. The decrease in SFA elicited by a given depolarizing current pulse was also accompanied by a marked reduction of the amplitude and duration of the postburst AHP as shown in Figure 5B + C.

These results are consistent with the idea that HZ application results, either directly or indirectly, in reduction of one or more  $I_{KCa^{++}}$ , which generate the AHP. This AHP blockade increases excitability in neurons exposed to HZs, and may contribute to epileptiform activity elicited by HZs in hippocampal neurons in slice and culture. In future experiments we plan to characterize the concentration dependence of HZ AHP-blocking effects, correlating this with concentration ranges of HZs capable of inducing epileptiform activity in hippocampal slices and cultures. As part of our Long Term Goals, we characterized HZ effects directly on single SK and BK channels, as well as on whole-cell voltage-clamp recordings of  $IKCa^{++}$ , to rigorously analyze the kinetics of HZ effects, and fully characterize HZ cellular mechanisms.

b) The Effects of HZs on Calcium Spikes and Calcium Currents.

Calcium-dependent AHP reduction by HZs is at least one mechanism by which these agents might increase excitability in hippocampal neurons. The AHP is generated secondarily following calcium entry into the neuron. Therefore, it is unclear whether HZ application is directly blocking the AHP conductance or reducing the AHP secondarily by blocking calcium entry into cells. We examined calcium spikes and the underlying calcium current, before, during exposure, and after washout of HZs to look for differences in calcium entry in hippocampal neurons following HZ exposure.

In preliminary experiments, we have recorded calcium spikes from cultured neurons following partial blockade of  $K^+$  currents with 5 mM 4-aminopyridine and 5 mM tetraethylammonium chloride, and blockade of sodium spikes with 1  $\mu$ M tetrodotoxin. Under these conditions, cultured hippocampal neurons fire large amplitude, long duration all-or nothing regenerative potentials following depolarization, and at the break of hyperpolarizing current commands (Figure 6). These findings are similar to previously published reports in hippocampal slices (42, 43) and cultures (18), and implicate a calcium-dependent regenerative "spike" as underlying these slow depolarizations. Studies proposed in this grant will examine the effects of HZ exposure on these slow depolarizing calcium spikes, to see if calcium entry is reduced by HZs, secondarily blocking calcium dependent AHPs.

In the event that HZ does affect calcium entry in cultured hippocampal neurons, we will need to examine HZ effects in greater detail under more rigorous conditions. We propose to use electrically compact enzymatically isolated hippocampal neurons to examine calcium current effects of HZs.

In preliminary experiments, we have dissociated CA1 neurons using a revised isolation process, and recorded 3 types of calcium currents from these cells. From a hyperpolarizing

holding potential (-100 mV), at lowest threshold, CA1 neurons exhibited a transient, inactivating 'T'-type calcium current. At higher threshold, inactivating and sustained components of calcium current were elicited, which may correspond to 'N'- and 'L'-type calcium currents (Figure 7; ref. 44). These currents were sufficiently large and stable under these recording conditions to permit detailed examination of short and longer term HZ effects on calcium currents in CA1 neurons. We propose in future experiments to examine HZ effects on all three types of calcium current present in these neurons.

## II. EFFECTS OF ANTICONVULSANTS ON THE EPILEPTOGENIC ACTION OF HZ

HZ-induced epileptiform activity in hippocampal slices is strikingly similar, both quantitatively and qualitatively, to epileptiform activity induced by bath application of the convulsant N-methyl-D-aspartate (NMDA). NMDA-induced epileptiform activity exhibits similar steep concentration dependence to that of HZ actions, increasing from threshold level effects to maximal effects within a one log unit increase in convulsant exposure (Figures 1 and 2; Figure 8; ref. 45). This steepness in concentration dependence suggests complex kinetics are involved in initiation of NMDA- and HZ-induced epileptogenic actions. Pharmacological complexities could, among myriad possibilities, include cooperative binding of more than one molecule of convulsant to a receptor involved in initiation of excitability increases, activation of multiple processes involved in response generation, or a combination of factors. In the case of NMDA, both processes are involved in generation of convulsant actions. Two molecules of NMDA are required to bind to the NMDA receptor to open the NMDA receptor complex ionophore, and  $Mg^{2+}$  voltage-dependent block must be alleviated before ions may permeate the open channel (46, 47). An additional parallel between HZ and NMDA actions is that NMDA exposure at higher concentrations results in a similar progression of excitability changes to that seen following higher concentration HZ application: an initial increase in stimulus-evoked

excitability, followed sequentially by spontaneous, prolonged epileptiform burst discharges, and a cessation of excitability (Figures 1 + 8; ref. 45). This striking similarity in actions of HZ to NMDA led us to hypothesize that HZ may act through activation of NMDA receptors, either primarily or secondarily via effects on other processes, to elicit its convulsant actions. As part of our longer term goals, we therefore propose to examine the effects of NMDA antagonists such as 2-amino-5-phosphonovaleric acid (APV) and MK-801 on HZ-induced epileptiform activity, in an initial attempt to explore the utility of various drugs in blocking HZ convulsant activity. In addition, we propose to examine the effects of various clinically utilized (and FDA approved) anticonvulsants on HZ-induced epileptiform activity, to develop effective therapeutic strategies which may reverse these effects. The anticonvulsants tested included diphenylhydantoin, carbamazepine, barbiturates, valproic acid, and anticonvulsant and sedative benzodiazepines.

### III. EFFECTS OF HZs ON SYNAPTIC MECHANISMS.

Preliminary evidence suggesting alternate convulsant mechanisms of HZ action, together with previously published reports suggesting that AHP and SFA effects are not sufficient to fully explain HZ actions in eliciting epileptiform discharges (for full discussion, see Experimental Design section), forces us, as a Long Term Goal, to search for further mechanisms of HZ action. We plan to focus these studies on HZ modulation of synaptic processes.

To accomplish this, we propose to use the newly developed slice patch technique (48-49) to record from hippocampal pyramidal neurons in slices during ongoing, HZ-induced epileptiform activity. We have already used the slice patch technique to examine synaptic and neuromodulatory responses in neurons from several brain areas, and as preliminary evidence, to demonstrate our ability to use this technique successfully, we present voltage-clamp recordings of spontaneous synaptic events recorded from a cortical pyramidal neuron (Figure

9), and a norepinephrine-evoked response in a thalamic neuron (Figure 10). The enhanced resolution recordings provided by this technique allowed detailed analysis of excitatory synaptic processes, before, during, and after HZ-induced epileptiform activity.

#### IV. SINGLE CHANNEL ANALYSIS OF HZ EFFECTS ON CALCIUM-DEPENDENT POTASSIUM CURRENTS

In preliminary experiments, we have been successful in applying single channel recording techniques to hippocampal neurons in culture and have accomplished both cell-attached and detached patch recordings of BK and SK channels. To demonstrate our ability to perform and to analyze data derived from this type of experiment, we present a brief example of some of our baseline characterization studies examining calcium-dependent potassium channels in cultured hippocampal neurons. Detached outside-out patch clamp recording revealed two types of  $\text{Ca}^{2+}$ -dependent outwardly conducting channels, with large and very small amplitudes (Figure 11).

The larger, high conductance channels had an amplitude of about 2 pA at a pipette potential of +10 mV. The amplitude of the channel increased linearly when the pipette potential was increased. The slope of this linearly increasing function yielded a single channel conductance for these channels of approximately 150 pS. Figure 11 shows examples of unitary channel activity at 3 pipette potentials. In the presence of  $10^{-8}$  M  $\text{Ca}^{2+}$  in the pipette (Figure 11B), these channels showed rapid openings and closings which often clustered into bursts of activity. The amplitude, the mean open-time, and burst length increased linearly with increasing pipette potentials. At this  $\text{Ca}^{2+}$  concentration the channels appeared to spend most of the time open within a burst. The channel activity was very much reduced when the pipette contained  $10^{-9}$  M  $\text{Ca}^{2+}$  (Figure 11A). In addition, the mean open-time and the burst length were also reduced. The kinetics and conductance (about 150 pS) of these channels are similar

to those of the BK subtype of  $I_{KCa++}$  reported in lactotrophs, which we have described previously (51), and in other vertebrate cell types (36, 37, 40, 41). The second calcium-dependent, outwardly conducting channel we encountered exhibited a much smaller amplitude unitary current and occurred less frequently, and probably corresponds to the SK channel described by others (36-39).

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#### **Professional Personnel Engaged in this Study**

Dr. Robert J. DeLorenzo has acted as the Principal Investigator in studying the effects of hydrazine on neuronal excitability. In addition, Dr. William W. Anderson and Dr. Sompong Sombati have developed the neuronal cells in culture. Dr. Douglas A. Coulter conducted the hippocampal slice experiments with Dr. Anderson.

#### **Inventions and Patents**

No specific inventions or patents have been developed in this research period.

#### **Summary**

During the 4th year of this research project, we have made considerable progress in

directing our efforts at studying the effects of hydrazine on neuronal excitability and on individual ion currents. The initial studies have been exciting and have led to research that will provide the first documentation of the effects of this compound on isolated neurons. In addition, the ability to incorporate experiments into this project that will also allow us to investigate the effects of hydrazine on vertebrate neurons considerably expands the scope of our initial goals. Development of this technology in our laboratory over the last year will greatly enhance the application and feasibility of these studies. We have also been successful in characterizing specific ion currents and their sensitivity to specific anticonvulsant and convulsant drugs. These studies will allow us now to more rigorously investigate mechanisms that may block the toxic effects of hydrazine on the nervous system.

This last year has allowed us to expand our sample sizes and rigorously document our papers for publication. This study has accomplished its specific aims and demonstrated direct effects of hydrazines on the central nervous system.